Fish and Shellfish Immunology 87 (2019) 714–720

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Spatiotemporal distribution of *Streptococcus agalactiae* attenuated vaccine strain YM001 in the intestinal tract of tilapia and its effect on mucosal associated immune cells

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ARTICLE INFO

Keywords:
S. agalactiae
Tilapia
Oral-gavage vaccination
Live attenuated vaccine

ABSTRACT

In this study, the tilapia was orally vaccinated by the attenuated *Streptococcus agalactiae*(*S. agalactiae*) strain YM001, and the distribution and the pathological effect of strain YM001 in different intestinal segments of tilapia were evaluated by real-time PCR(qPCR), immunohistochemistry(IHC) and histomorphology. The qPCR results showed that the number of bacteria was the highest in the intestinal tracts at 12 h post oral gavage in the YM001 group, then began to decrease sharply and eliminated at 7 d. And the number of bacteria was highest in the foregut, hindgut, and rectum at 12 h, 24 h, and 3 d, respectively. IHC indicated that bacteria mainly distributed in the margin epithelium and the goblet cells at 12 h - 24 h, and in the submucosa and muscle layer in the YM001 group in 3 d post gavage, then almost disappeared at 7 d. Histological examination of intestines post gavage displayed that an inflammation was observed at 7 d in the YM001 group and the intestinal structure was fully recovered at 15 d. and the intestinal structure was fully recovered at 15 d. Conclusion: The attenuated *S. agalactiae* vaccine strain YM001 could enter the intestinal tissue after oral gavage and had a strong spatial and temporal selectivity in the intestinal tract, which could cause obvious mucosal immune response and mild pathological reaction, but the pathological change could be gradually repaired with the extinction of bacteria in the body.

1. Introduction

S. agalactiae is one of the major pathogens of tilapia, which causes huge economic losses to the tilapia industry every year [1–4]. Vaccine is one of the effective methods to prevent and control the outbreak of *S. agalactiae* [5]. So far, many studies have been focused on the development of vaccines against *S. agalactiae*-caused disease and have achieved good immune effects [6–13]. These studies include inactivated vaccines [6–11], vaccines with bacterial extracellular products [7,9,11], the subunit vaccine [8,13], and other types vaccines. The immunization methods used for these vaccines include injection [6,7,9,11], immersion [6], spraying [10], and oral administration [8,12,13]. Immunization by injection route results in the best protection, but is usually labor-intensive and causes stress in fish, thus it is difficult to apply in large scale. Immersion is suitable for immunizing small fish, but the effect is not promising [6]. In contrast, oral vaccine is easy to operate and cause less stress in fish, and is most suitable for large-scale fish population immunization. However, because the antigens are difficult to reach the immune organs, after orally administrating the inactivated vaccines and subunit vaccines, this method almost cannot effectively induce the systemic immune response, and can only generate short-term local immune response in the intestinal tract, usually within 7 days [13]. Additionally, the local mucosal immune response elicited by inactive antigens is relatively weak and does not achieve a satisfactory protection efficacy [6,14]. Oral attenuated vaccines have better effects than oral inactivated vaccines, mainly because oral attenuated vaccines use live bacteria as antigens, which can avoid degradation by intestinal pancreatic enzymes, and reach the immune organs such as spleen and kidney, inducing a long-term immune response [15]. Furthermore, oral attenuated vaccines can induce both systemic and local immune responses [16,17]. Therefore, development of oral attenuated live vaccine for tilapia *streptococcosis* is very important to prevent the occurrence of this disease.

In this work, *S. agalactiae* attenuated vaccine YM001 could be largely detected in the brain, liver and spleen of tilapia after intragastric administration [18]. However, there are still many questions that need

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https://doi.org/10.1016/j.fsi.2019.02.003
Received 11 October 2018; Received in revised form 31 January 2019; Accepted 1 February 2019
Available online 07 February 2019
1050-4648/

to be further studied. For example, how the attenuated live vaccine enters the intestine? how it is distributed in the intestine? and whether it effectively induces mucosal immune response? In the present study, histomorphology, IHC and qPCR were employed to study the spatial distribution of vaccine strain in tilapia intestine, as well as histomorphological changes in the intestine after gavage of the attenuated vaccine strain YM001. It explored the possible mechanisms how YM001 broke through the intestinal mucosal barrier, and provided a theoretical basis for further optimizing the YM001 oral vaccine and developing other fish oral vaccines.

2. Materials and methods

2.1. Bacterial strains and fish

S. agalactiae HN016 was isolated from an outbreak epidemical disease in tilapia from Hainan, China in 2010 [19]. The strain HN016 was continuously passaged in vitro, until obtaining a strain YM001, after 840 passages [18]. The bacterial strains had been stored at −80 °C.

Non-infected Nile tilapia with average weight of 155.45 ± 20.85 g was provided by the National Tilapia Seed Farm (Nanning, Guangxi, China). Prior to experiments, 50 fish were acclimated in fiberreinforced plastic tanks (800 L each, with 3 replicates) with a stocking rate of 4 g/L under 30 ± 4 °C for 2 weeks. The experimental fish were confirmed to be negative for bacterial infection by bacteriological analysis of the brain and kidney samples. All the tanks were equipped with separate recirculation system with external biofilters (Haisheng, China). Fish were fed twice a day with a formulated diet (Tongwei Feed Company, Nanning, China). All the experiments were conducted according to the principles and procedures of the Laboratory Animal Management Ordinance of China [18]. In the experiment, the fish were immersed in 100 ng/ml tricaine methanesulphonate (MS-222, Solarbio, China) for anesthetization and in 300 ng/ml MS-222 at least 10 min for euthanasia.

2.2. Oral gavage

The stored HN016 and YM001 were thawed, streaked onto 5.0% sheep blood agar plates, and incubated at 28 °C for 24 h. Then single colony of every strain was inoculated in TSB and incubated at 28 °Cfor 24 h under low agitation. The concentration of the bacteria was determined using colony forming unit (CFU) per mL by plating 100 μL of 10-fold serial dilutions onto blood agar plates.

Before oral gavage, the fish were anesthetized with MS222. Fish were given HN016 or YM001 at the dose of 1.0×10^9 CFU/fish and 1.0×10^9 CFU/fish, respectively, and the control group was treated with 1.0 mL of TSB. The infected fish were monitored and fed twice a day for 30 days, and the bacteria were isolated from the brain and liver tissues of fish in YM001 group at 1 d and all dead fish after gavage, and identified.

2.3. Histology

At 12 h, and 1, 3, 7 and 15 days, respectively, after the oral gavage, 5 fish in each group were randomly removed to histological examination. The intestinal samples (Fig. 1) were collected and fixed in 10% neutral-buffered formalin for 24 h. The samples were sequentially dehydrated by passage through a gradient of ethanol solutions and embedded in paraffin wax. Six μm thick sections were cut and then stained with hematoxylin-eosin (H&E) for tissue morphology or Alcian blue/periodic acid–Schiff (AB-PAS) for goblet cell count. The numbers of intraepithelial leucocytes (IELs) and goblet cells in the epithelium (defined as the region between the lamina propria and the microvilli brushborder), across a standardized distance of 100 μm, were then calculated by averaging the cell numbers from all specimens [20].

2.4. qPCR

Total DNA was extracted from samples by using TIANamp Genomic DNA Kit (Tiangen, China) according to the manufacturer's instructions and stored at −20 °C until qPCR amplification. The amount of bacteria was detected by using the Taqman absolute quantitative PCR method that established in our lab for *S. agalactiae.* Briefly, the forward primer was: cfb-F (5'→3′) CGGTTAATGAGGCTATTACTAGTG; the reverse primer was: cfb-R (5'→3′) ATCTGTTAAGGCTTCTACACGAC; the probe was: cfb-P (5'→3′) FAM-TTCATTGCGTGCCAACCCTGAGACA-Eclipse. The size of the amplified product was 113 bp. The primers were synthesized in Takara (Dalian, Japan). The detection was performed with Premix Ex TaqTM kit (Takara, Dalian, Japan) by using the German Analytik Jena qTOWERE 2.2 real-time PCR instrument. The number of *S. agalactiae* in the fish tissues of the three parallel groups at each sampling time point from the experimental groups were determined according to the standard curve, and the average value was calculated. The reaction system was Premix Ex Taq 25.0 μl, Forward Primer 1.0 μl, Reverse Primer 1.0 μl, TaqMan Probe 2.0 μl, ROX Reference Dye II 0.5 μl, DNA template 4.0 μl, DNase/RNase-free water 16.5 μl, total volume was 50 μl. The reaction procedure was: 95 °C 30 s; 95 °C 5 s, 60 °C 30 s, 40 cycles. PCR product was sent to Bao BioengineeringCo., Ltd. (Dalian, Japan) for sequencing.

2.5. IHC

Six μm thick sections were cut and incubated 37 °C for 24 h and then incubated overnight at 60 °C Paraffin embedded tissue slides were deparaffinized with xylene, rehydrated, and unmasked following standard immunohistochemical methods. Heat-induced antigen retrieval was performed in citrate buffer (10 mM trisodium citrate in water, pH 6) for 10 min at 100 °C [21]. Slides were left to cool for 15 min and subsequently washed. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 15 min followed by washing in 10 mM PBST for 5 min. Blocking of non-specific sites was achieved by incubation in 5% bovine serum albumin (BSA) in PBST for 30 min. The section was treated with the primary antibody (a rabbit polyclonal antiserum raised against *S. agalactiae*) at a dilution of 1:400 (sterile PBS, pH 7.4) for over night at 4 °C in ahumidchamber. Section was washed with PBST and treated with goat anti-rabbit horseradish peroxidase (HRP) conjugate (Sigma, USA) at a dilution of 1:100 for 30 min at 37 °C. The antibody bound proteins were then visualized by DAB-H_2O_2. The slide was observed

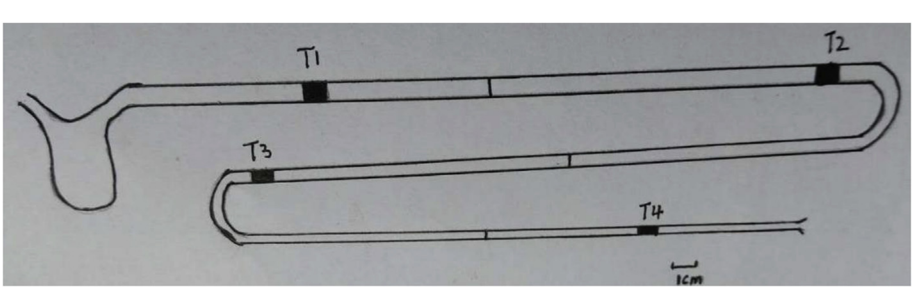

Fig. 1. Schematic drawing of the Nile tilapia (*O. niloticus*) digestive tract in which the intestinal segments sampled for histology and immunohistochemistry were indicated. The four intestinal segments were sampled every ~1 cm along the intestine. Segments T1 corresponded to duodenum, Segments T2 to foregut, Segments T3 to hindgut, while T4 were collected from rectum. Bar = 1 cm.

under the microscope (Olympus, Japan). Negative controls for all samples were done using the secondary antibody alone.)

2.6. Experimental S. agalactiae challenge test

On the 15th day after oral gavage, the bacteria culture of *S. agalactiae* virulent strain HN016 was used to challenge the fish by intraperitoneal injection with a dose 5.24 sa^6 CFU/fish [18]. The fish were observed for 15 days after challenge, and the feeding, morbidity and mortality were recorded every day. The bacteria from brain and liver of the dead fish were isolated and cultured using blood agar plate, and calculated the relative immune protection rate.

2.7. Statistical analysis

All the data were presented as mean ± S.D. from three replicates and analyzed by one-way ANOVA with Duncan method using software SPSS Statistics 17.0. The significance level was defined as $P < 0.05$.

3. Results

3.1. Disease symptoms and bacterial isolation of experimental fish after oral gavage

In the YM001 group, the food intake of the experimental fish was reduced at 24–72 h after oral gavage, and no other changes were observed. After 4 days, the appetite returned to normal. There was no dead fish in YM001 group during 15 d after oral gavage. But in the HN016 group the typical symptoms of *streptococcal* infection occurred: slow swimming, abnormal posture, black body at 6 h, occurrence of death at 12 h, and death of most fish at 24 h, almost 100% death at 3 d. *S. agalactiae* can be isolated from the brain and liver of the dead fish.

The results of bacterial culture isolated at 24 h after oral gavage in YM001 group was that most of blood agar plates (60%) had small amount of colonies (less than 50 colonies), and a few showed dense growth (20%, it is difficult to distinguish single colonies), and rest of plates had no bacterial growth (20%). In contrast, bacteria isolated from the HN016 group showed dense growth on all plates.

The relative percent survival of tilapia was 75.44% at 15 d after *oral gavage* in YM001 group (Table .1).

3.2. Results of qPCR

In the YM001 group, the number of bacteria was the highest in the intestinal tracts of the experimental fish at 12 h, and the number of bacteria began to decrease sharply at 24 h, and no bacteria were detected at 7 d (Fig. 2). Among them, the number of bacteria in the foregut was up to 5.4×10^7 cfu at 12 h. The highest number of bacteria was detected in the foregut at 12 h, and then detected in hindgut at 24 h, and finally was detected in the rectum at 3 d.

In HN016 group, the number of bacteria in each intestinal segment was 10^4-10^5 cfu at 12 h, and the number of bacteria was reduced by an

Table 1
Mean mortality rate and relative percent survival of vaccinated tilapia challenged with virulent *Streptococcus agalactiae*.

Test group	No. of. fish challenged	No. of fish deaths	Mortality rate	Mean mortality rate	Relative percent survival
YM001	20	4	20	23.33	75.44
	20	5	25		
	20	5	25		
control	20	19	95	95	–
	20	18	90		
	20	20	100		

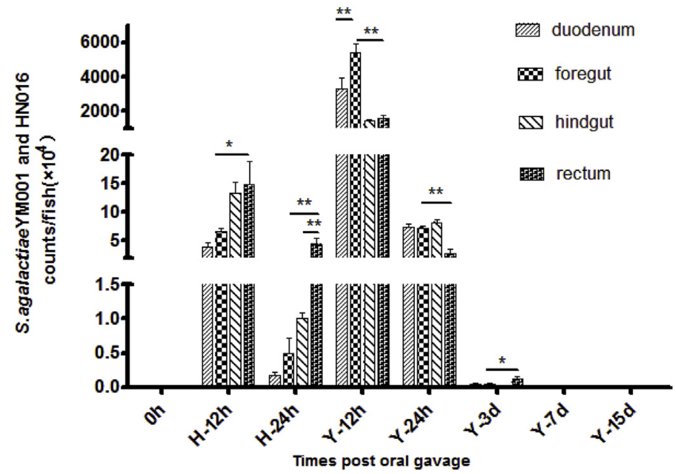

Fig. 2. Live attenuated *S. agalactiae* loads in the intestine of tilapia. Tilapia were challenged by oral gavage with 10^9 CFU ml^{-1} *S. agalactiae* YM001 (Y) and HN016 (H). Total DNA of each sample from five tilapia was extracted for qPCR analysis. Ct values were converted into *S. agalactiae* YM001 and HN016 counts. Bars represents the mean counts of three biological replicates and the error bars represents standard deviations. Statistical significance was analyzed between bacterial counts at 12 h, 1 d, 3 d, 7 d, 15 d h p. v. and 0 h p. v. in each tissue (*$P < 0.05$, **$P < 0.01$). 0 h was served as a control, in which *S. agalactiae* was not detected. *S. agalactiae* was not detected at 7 and 15 d.

order of magnitude at 24 h, and minimum reduction was observed in the rectum. The number of bacteria was increased from duodenum to rectum, and the highest amount of bacteria was detected in rectum at 12 h and 24 h.

In the YM001 group, the number of bacteria in the duodenum and foregut of the experimental fish was significantly higher than that in the hindgut and rectum ($P < 0.05$) at 12 h, and the number of bacteria in the anterior three segments of intestine was significantly higher than that in the rectum ($P < 0.05$), whereas the HN016 group showed opposite results. At 3 d, the highest bacterial amount was detected in the rectum in YM001 group, which was significantly higher than other three segments of intestine ($P < 0.05$).

3.3. Histomorphology

Sections of intestines from the tilapia were stained using H&E to examine pathological changes induced by *S. agalactiae* YM001. It was showed the four segment intestine from tilapia with an intact edge of villi in the control group (Fig. 3A–D). In the YM001 group, the goblet cells in foregut and rectum increased visibly at 12 h (Fig. 3J) and lasted 3 d, and then there were many vacuoles in the villi at 7 d (Fig. 3K). And lymphocytes were observed distributing sporadically in lamina propria at 12–24 h. Later, some of lymphocytes transported to the surface of mucous layer at 3 d, broke through the epithelial barrier, and infiltrated to the lumen at 7 d (Fig. 3K-L). Moreover, the serosa layer was slightly thickened and mild edema occurred. Finally, the physiological status of intestine returned to the normal conditions at 15 d. These results confirmed that inflammatory responses were induced by *S. agalactiae* YM001 in the intestine, lasting no more than 15 d.

In the HN016 group, the inferior borders of each intestinal segment were observed to be unclear, broken, and shedding (Fig. 3E–H). A large number of necrotic and exfoliated epithelial cells were observed in the intestinal lumen, and the structure of lamina propria and submucosa was loose and vacuoles were observed. Furthermore, the muscular layers and serosal layer were thickened and edema occurred (Fig. 3G–H).

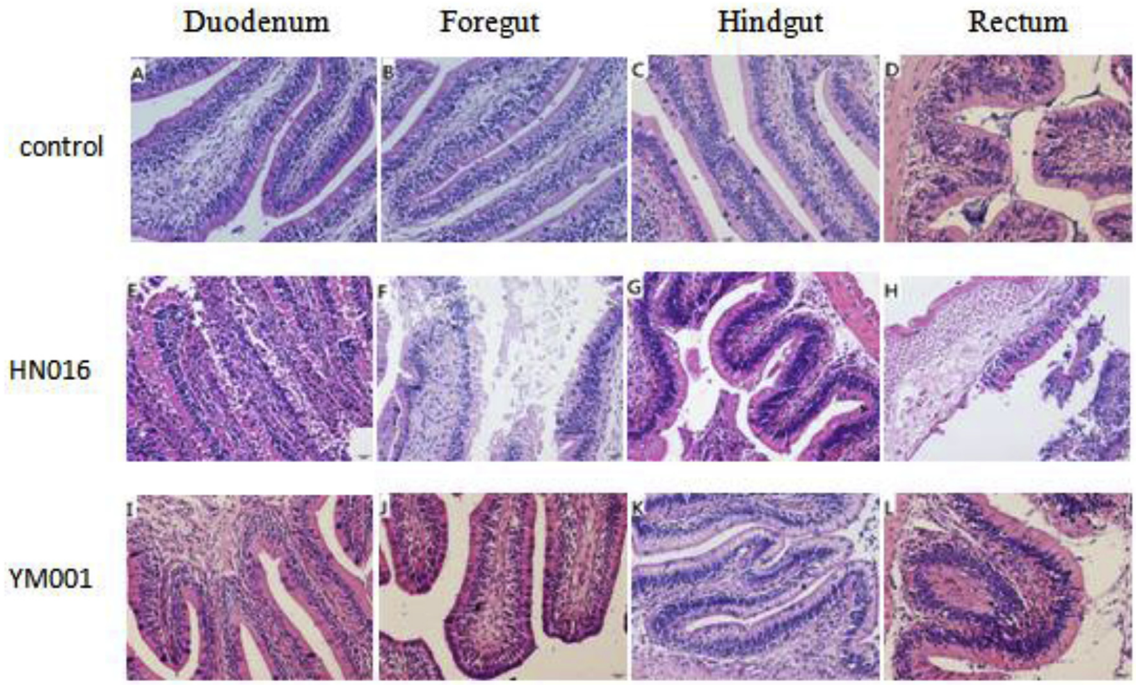

Fig. 3. Inflammatory responses induced by *S. agalactiae* YM001 (Y) and HN016 (H) in the intestine of tilapia after oral gavage. Tilapia were challenged by oral gavage with 10^9 CFU ml^{-1} *S. agalactiae* YM001 and HN016. Intestine of tilapia was sampled and fixed in 10% neutral-buffered formalin for histology analysis. The line represents 50 μm length under 200× magnification.

3.4. Goblet cells

In the YM001 group, the manifestations of each intestinal segment were different (Fig. 4 and Fig. 5I-5L). The number of goblet cells in the duodenum, hindgut and rectum was increased first and then decreased, and was lowest at 7 d, and then returned to the level of control group at 15 d. The number of goblet cells in the duodenum, hindgut and rectum reached the highest values at 3 d, 3 d and 24 h, respectively, while the number of goblet cells in the foregut reached the highest value at 12 h and then began to decrease, and recovered to the level of the control group at 15 d.

In HN016 group, the number of goblet cells in each intestinal segment was lower than that of the control group ($P < 0.05$) (Fig. 4 and Fig. 5E–H). The number of goblet cells in the foregut, hindgut and rectum linearly decreased, while the cell number in duodenum decreased first and then increased.

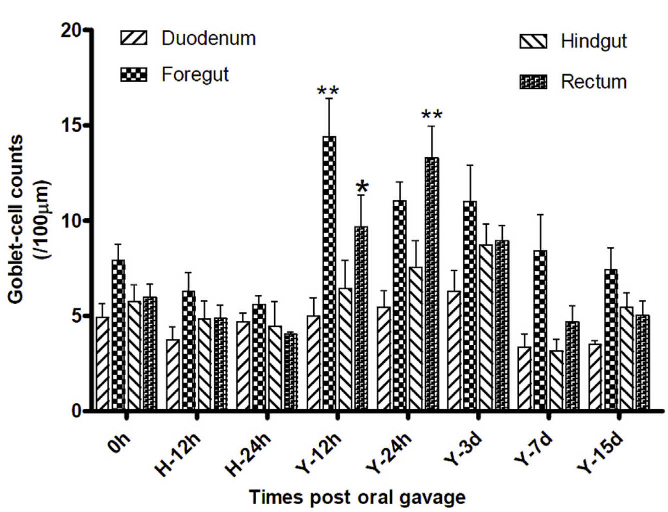

Fig. 4. The number of goblet cells in tilapia intestinal tracts was statistically analyzed based on three separate samples and Student's *t*-test was used to detect the significant difference between the control and oral gavage groups, $^{*}P < 0.05$,$^{**}P < 0.01$. H:HN016,Y:YM001.

The number of goblet cells in the foregut from two experimental groups was the highest at each time point (except the YM001 group that occurred at 24 h); the number of goblet cells in the foregut (12 h) and rectum (12 h, 24 h) of the YM001 group was significantly higher than that of the control group ($P < 0.05$). The number of goblet cells in the duodenum and hindgut increased slightly and then decreased, but the change was small.

3.5. IELs

In the YM001 group, the number of IELs in the duodenum decreased at 12 h (Fig. 6), and then increased to the highest value at 3 d. The number of IELs in the foregut remained at a high level from 24 h to 7 d, and then decreased to the level of the control group. In the hindgut, the IELs reached a high value at 12 h, 3 d, and 15 d. In the rectum, the IELs decreased slightly, then reached the highest value at 3 d, and then decreased to the control level.

In the HN016 group, the number of IELs in the duodenum decreased to a level of the control group after a slight increase at 12 h (Fig. 6). The IELs in the foregut began to decrease at 24 h, whereas the number of IELs in the hindgut showed an upward trend. In the rectum, the IELs decreased slightly at 12 h and then increased.

3.6. Results of IHC

In the YM001 group, higher numbers of immunohistochemically positively stained bacteria distributed in intestinal villus margin and the goblet cells at 12 h - 24 h after oral gavage (Fig. 7I and J). Later, higher numbers of bacteria concentrated in the submucosa and muscle layer of the intestine at 3 d (Fig. 7K-L), and there were no positive stained bacteria at 7 d. No positive stained bacteria were detected of the control group (Fig. 7A–D).

In the HN016 group, a large amount of positive stained bacteria was

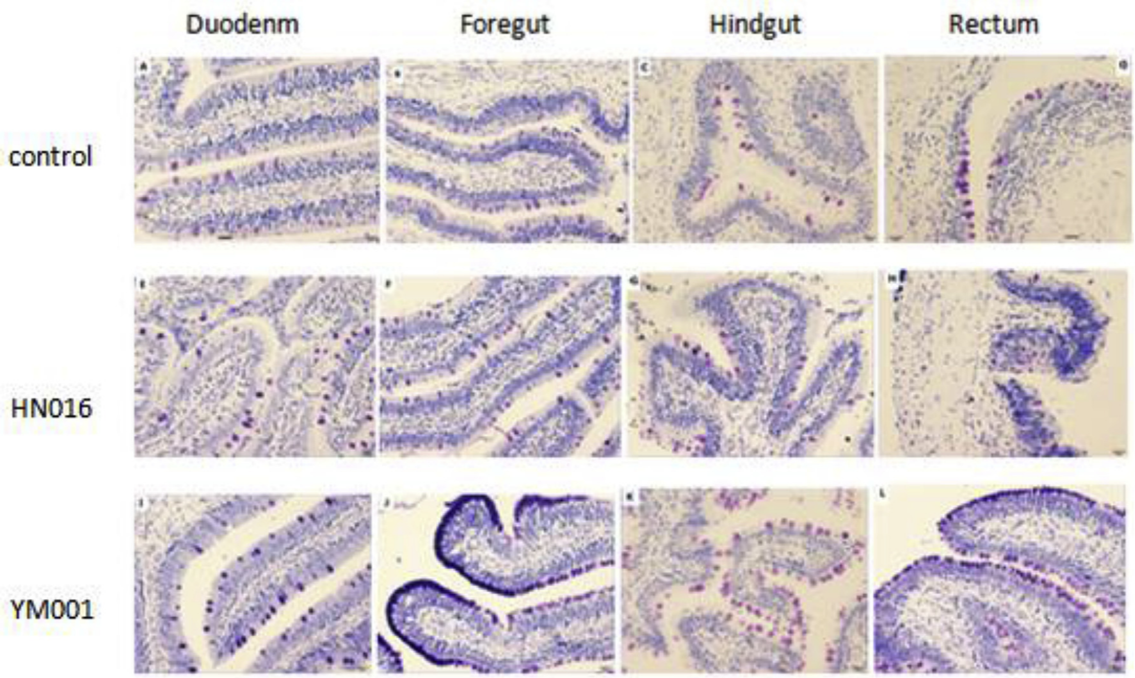

Fig. 5. Histological analysis of goblet cells in intestine of tilapia in control and oral gavage groups. In PAS/AB staining, Fuchsia indicated the goblet cells. The sampling time of HN016 group and YM001 group was 24 h. The line represents 20 μm length under 400× magnification.

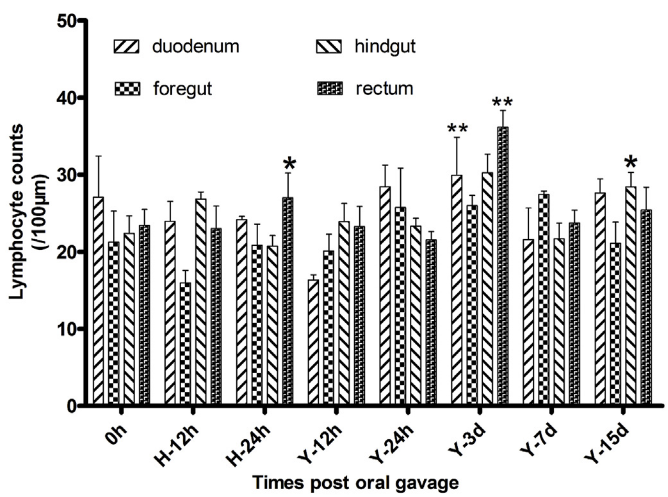

Fig. 6. Lymphocyte counts in the intestine of tilapia oral gavage with *S. agalactiae* YM001 (Y) and HN016 (H). Tilapia were challenged by oral gavage with 10^9 CFU ml^{-1} *S. agalactiae* YM001 and HN016. Lymphocyte cells were directly evaluated by light microscope at various time points based on histological sections. Each bar represented the mean of three biological replicates and error bars represented standard deviation. Statistical significance was analyzed between the oral gavage and control groups of tilapia (*P < 0.05, **P < 0.01).

observed at 12 h in the duodenum, foregut, hindgut and rectum (Fig. 7E and H), and mainly concentrated in the villus, submucosa and muscle layer of the intestinal mucosa. Positive stained bacteria were observed in the submucosa and muscle layers at 24 h (Fig. 7F and G). The difference between the YM001 group and the HN016 group was that the stained goblet cells of the former intestine were darker (Fig. 7J), while the latter goblet cells were often not stained (Fig. 7E–H).

4. Discussion

In the aquaculture industry, oral immunization have more advantages than injection immunization, while attenuated live vaccines are more suitable for oral and immersion immunization because of their better immunogenicity and longer immune protection [22], but more attention should be paid for the safety of live vaccine. Huang et al. used live attenuated *Salmonella typhimurium* as carrier to produce attenuated vaccine expressing *S. agalactiae* surface antigen (Sip); however, the attenuated strain produced by this method displayed a poor stability and a weak immune response, and can only last for 7 days by oral immunization [23]. Li et al. [18] found that after administration of tilapia with attenuated *S. agalactiae* strain YM001, *S. agalactiae* can be detected at 6 h in the brain, kidney, spleen, and liver of fish. *S. agalactiae* was not detectable in the brain, kidney and liver after 3 d, 5 d and 11 d, respectively, while a small number of bacteria still can be detected in the spleen at 15 d. In this study, the number of *S. agalactiae* in intestine reached 10^7 CFU at 12 h after gavage of tilapia with attenuated *S. agalactiae* vaccine strain YM001 at a dose of 10^9 CFU/fish, and the bacterial number began to decrease sharply at 24 h, but it was still detectable at 3 d. At 7 d, there was no any detected *S. agalactiae* the intestinal tract. Notably, the experimental fish did not die during 15 d after gavage. These results indicated that the attenuated vaccine strain YM001 could break through the intestinal mucosal barrier and reach the whole body tissues through the intestinal blood circulation. Although it can survive in the body for a short period of time, which will eventually be cleared. In contrast, the experimental fish in the HN016 group showed symptoms at 6 h, and death occurred at 12 h. Most of fish died at 24 h and almost 100% died at 3 d. These results indicated that the damage caused by the virulent strain HN016 to the fish was irreversible and irreparable. It has been reported that epithelial cells, IELs, and eosinophils are involved in intestinal inflammation [24]. Histomorphology results also showed that goblet cells and intraepithelial lymphocytes in the same intestinal segment increased successively or simultaneously; the intestinal villi were damaged, the epithelial cells were shed into the intestinal lumen, and there were many vacuoles in the villi, and the serosa layer was slightly thickened at 7 d; and the intestinal structure was fully recovered at 15 d. In the HN016 group, it was observed that the inferior borders of each intestinal segment was unclear, broken, and shedding. In addition, the number of goblet cells

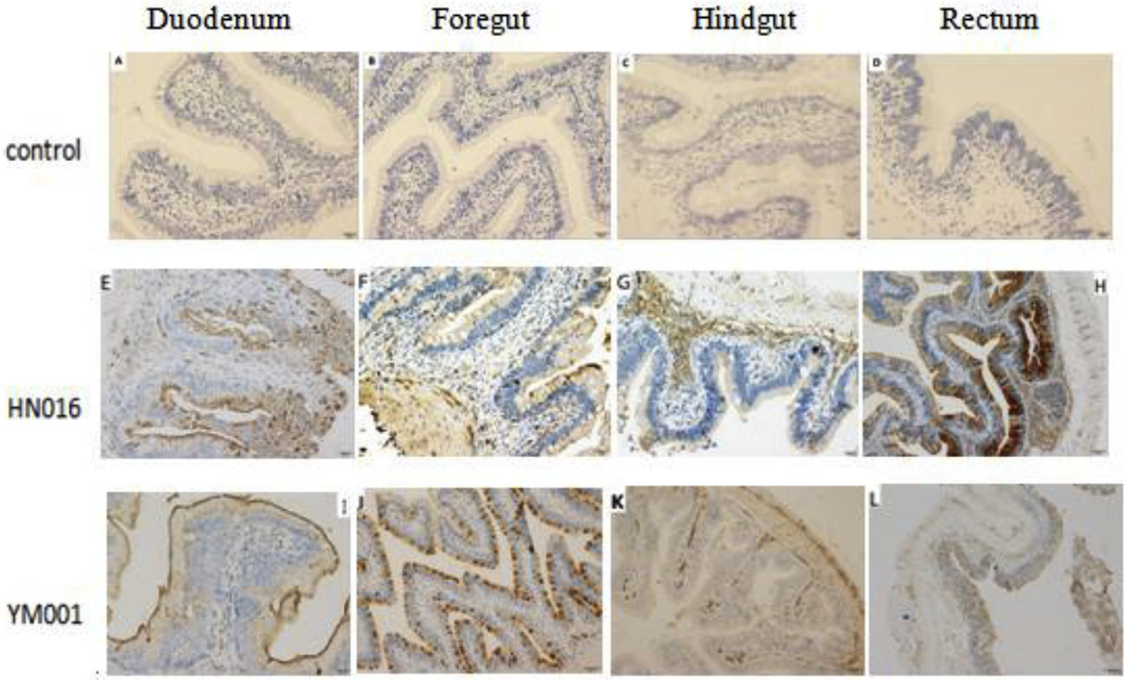

Fig. 7. Immunohistochemistry (IHC) of tilapia gut challenged by oral gavage with *S. agalactiae* YM001 and HN016 (400×). Widespread positive immune-stained bacteria (brown color) (E–L) compared to none immune-stained intact bacteria (A–D), respectively. G-H and J-L were 200×, A- F and I Were 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and IELs in the intestine of YM001 group also changed successively, but both type cells recovered to near normal levels, suggesting that although YM001 induced slight pathological reaction of intestinal tissue, it can be recovered gradually with the disappearance of bacteria in the body, thus which has good safety. Similarly, Liu et al. [25] also found that the bacterial number from the intestinal tract of zebrafish that immersed with the attenuated *Vibrio anguillarum* vaccine MVAV6203 was the highest at 3 h, but it could not be detected at 24 h. In the meantime, the intestinal mucosa showed slight shedding at 12 h, and which gradually repaired at 24 h. Moreover, the IELs were increased from 6 h and reached a peak at 12 h, and dropped to the control level at 120 h, and then the goblet cells began to increase.

The direct contact of the skin, gill and intestines of the bony fish with the external environment has become the main route for aquatic pathogens to enter the host [25,26]. Oral vaccination or anal perfusion methods can also cause an intestinal immune response and produce effective immune protection [27]. Intestinal uptake of antigen occurs mainly in the second half of the fish's intestine, accounting for 20%–25% of the total length of the intestine. The epithelial cells of this intestinal segment can uptake large antigens by endocytosis and transfer them to macrophages of the lamina propria and the blood circulation system [28,29]. Some researchers reported that the midgut was an important tissue for the uptake and presentation of antigens by the fish mucosal immune system [30]. The present study found that the number of bacteria from the intestinal segments of the YM001 group was the highest at 12 h, and the bacterial number in foregut was up to 5.4×10^7 CFU. The bacterial amount began to decrease significantly at 24 h, and was still detectable at 3 d, but the bacteria was not detected at 7 d. The distribution of bacterial number in each intestinal segment was as follows: the highest amount of bacteria was detected in the foregut, hindgut, and rectum at 12 h, 24 h, and 3 d, respectively. The results of IHC showed that bacteria mainly distributed in the margin epithelium and the goblet cells at 12 h - 24 h after oral gavage, and in the submucosa and muscle layer in the YM001 group in 3 d post gavage, then almost disappeared at 7 d. These results indicated that after the gavage, the number of the attenuated vaccine strain YM001 in the intestine was gradually decreased until they all died, and the distribution of YM001 in the intestine had certain spatial and temporal selectivity.

5. Conclusion

The attenuated *S. agalactiae* vaccine strain YM001 can enter the intestinal tissue after gavage, and their distribution in the intestinal tract showed strong spatial and temporal selectivity. YM001 can cause obvious mucosal immune response and mild pathological reaction, but which can be gradually repaired with the extinction of bacteria in the body.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

This work was supported by National Natural Science Foundation of China, China (Grant no. 31460695), Guangxi innovation-driven development special fund, China (Grant no. AA17204081-3), China Agriculture Research System, China (CARS-46), Guangxi Natural Science Foundation, China (Grant no. 2016GXNSFDA380020), Guangxi Key Laboratory for Aquatic Genetic Breeding and Healthy Aquaculture, China (2018), Guangxi public welfare research, China (Grant no. 2060302 CXIF-2016-08).

References

[1] J.J. Evans, P.H. Klesius, D.J. Pasnik, J.F. Bohnsack, Human *Streptococcus agalactiae* isolate in Nile tilapia(Oreochromis niloticus), Emerg. Infect. Dis. 15 (5) (2009) 774–776.

[2] J.C. Garcia, P.H. Klesius, J.J. Evans, C.A. Shoemaker, Non-infectivity of cattle *Streptococcus agalactiae* in Nile tilapia, Oreochromis niloticus and channel catfish, Ictalurus punctatus, Aquaculture 281 (1) (2008) 151–154.

[3] U.P. Pereira, G.F. Mian, I.C. Oliveira, L.C. Benchetrit, G.M. Costa, H.C. Figueiredo, Genotyping of *Streptococcus agalactiae* strains isolated from fish, human and cattle and their virulence potential in Nile tilapia, Vet. Microbiol. 140 (1–2) (2010)

186–192.
[4] J.W. Pridgeon, P.H. Klesius, Development and efficacy of a novobiocin-resistant Streptococcus iniae as a novel vaccine in Nile tilapia (Oreochromis niloticus), Vaccine 29 (35) (2011) 5986–5993.
[5] A.M. Darwish, Laboratory efficacy offlorfenicol against Streptococcus iniae infection in sunshine bass, J. Aquat. Anim. Health 19 (1) (2007) 1–7.
[6] J.J. Evans, P.H. Klesius, C.A. Shoemaker, Efficacy of *Streptococcus agalactiae* (group B) vaccine in tilapia (Oreochromis niloticus) by intraperitoneal and bath immersion administration, Vaccine 22 (27–28) (2004) 3769–3773.
[7] D.J. Pasnik, J.J. Evans, V.S. Panangala, P.H. Klesius, R.A. Shelby, C.A. Shoemaker, Antigenicity of *Streptococcus agalactiae* extracellular products and vaccine efficacy, J. Fish. Dis. 28 (4) (2005) 205–212.
[8] M. Nur-Nazifah, M.Y. Sabri, A. Siti-Zahrah, Development and efficacy of feed based recombinant vaccine encoding the cell wall surface anchor family protein of *Streptococcus agalactiae* against streptococcosis in Oreochromis sp, Fish Shellfish Immunol. 37 (1) (2014) 193–200.
[9] D.J. Pasnik, J.J. Evans, P.H. Klesius, Passive immunization of Nile tilapia (Oreochromis niloticus) provides significant protection against *Streptococcus agalactiae*, Fish Shellfish Immunol. 21 (4) (2006) 365–371.
[10] O. Noraini, M.Y. Sabri, A. Siti Zahrah, Efficacy of spray administration of formalin-killed *Streptococcus agalactiae* in hybrid Red Tilapia, J. Aquat. Anim. Health 25 (2) (2013) 142–148.
[11] D.J. Pasnik, J.J. Evans, P.H. Klesius, Duration of protective antibodies and correlation with survival in Nile tilapia Oreochromis niloticus following *Streptococcus agalactiae* vaccination, Dis. Aquat. Org. 66 (2) (2005) 129–134.
[12] C.A. Shoemaker, G.W. Vandenberg, A. Désormeaux, P.H. Klesius, J.J. Evans, Efficacy of a Streptococcus iniae modified bacteria delivered using OraljectTM technology in Nile tilapia (Oreochromis niloticus), Aquaculture 255 (1) (2006) 151–156.
[13] L.Y. Huang, K.Y. Wang, D. Xiao, D.F. Chen, Y. Geng, J. Wang, Y. He, E.L. Wang, J.L. Huang, Xiao GY,Safety and immunogenicity of an oral DNA vaccine enco ding Sip of *Streptococcus agalactiae* from Nile tilapia Oreochromis niloticus delivered by live attenuated Salmonella typhimurium, Fish Shellfish Immunol. 38 (1) (2014) 34–41.
[14] G.W. Vandenberg, Oral vaccines for finfish: academic theory or commercial reality? Anim. Health Res. Rev. 5 (2) (2004) 301–304.
[15] W.H. Chen, J. Garza, M. Choquette, J. Hawkins, A. Hoeper, D.I. Bernstein, M.B. Cohen, Safety and immunogenicity of escalating dosages of a single oral administration of Peru-15 pCTB, a candidate live, attenuated vaccine against enterotoxigenic Escherichia coli and Vibrio cholerae, Clin. Vaccine Immunol. 22 (1) (2015) 129–135.
[16] M. Makesh, P.S. Sudheesh, K.D. Cain, Systemic and mucosal immune response of rainbow trout to immunization with an attenuated Flavobacterium psychrophilumvaccine strain by different routes, Fish Shellfish Immunol. 44 (1) (2015) 156–163.
[17] J.B. Locke, R.K. Aziz, M.R. Vicknair, V. Nizet, J.T. Buchanan, Streptococcus iniae M-like protein contributes to virulence in fish and is a target for live attenuated vaccine development, PLoS One. 3 (7) (2008) 2824–2829.
[18] L.P. Li, R. Wang, W.W. Liang, T. Huang, Y. Huang, F.G. Luo, A.Y. Lei, M. Chen, X. Gan, Development of live attenuated *Streptococcus agalactiae* vaccine for tilapia via continuous passage in vitro, Fish Shellfish Immunol. 45 (2) (2015) 955–963.
[19] M. Chen, R. Wang, L.P. Li, W.W. Liang, J. Li, Y. Huang, A.Y. Lei, W.Y. Huang, X. Gan, Screening vaccine candidate strains against *Streptococcus agalactiae* of tilapia based on PFGE genotype, Vaccine 30 (42) (2012) 6088–6092.
[20] R.M.W. Ferguson, D.L. Merrifield, G.M. Harper, M.D. Rawling, S. Mustafa, S. Picchietti, J.L. Balcázar, S.J. Davies, The effect of Pediococcus acidilactici on the gut microbiota and immune status of on-growing red tilapia (Oreochromis niloticus), J. Appl. Microbiol. 109 (3) (2010) 851–862.
[21] C.J. Chang, C. Robertsen, B. Sun, B. Robertsen, Protection of Atlantic salmon against virus infection by intramuscular injection of IFNc expression plasmid, Vaccine 32 (2014) 4695–4702.
[22] J. Frey, Biological safety concepts of genetically modified live bacterial vaccines, Vaccine 25 (30) (2007) 5598–5605.
[23] L.Y. Huang, K.Y. Wang, D. Xiao, D.F. Chen, Y. Geng, J. Wang, Y. He, E.L. Wang, J.L. Huang, G.Y. Xiao, Safety and immunogenicity of an oral DNA vaccine enco ding Sip of *Streptococcus agalactiae* from Nile tilapia Oreochromis niloticus delivered by live attenuated Salmonella typhimurium, Fish Shellfish Immunol. 38 (1) (2014) 34–41.
[24] J.H. Rombout, L. Abelli, S. Picchietti, G. Scapigliati, V. Kiron, Teleost intestinal immunology, Fish Shellfish Immunol. 31 (5) (2011) 616–626.
[25] X. Liu, H. Wu, X. Chang, Y. Tang, Q. Liu, Y. Zhang, Notable mucosal immune responses induced in the intestine of zebrafish (Danio rerio) bath-vaccinated with a live attenuated Vibrio anguillarum vaccine, Fish Shellfish Immunol. 40 (1) (2014) 99–108.
[26] J. Corrales, I. Mulero, V. Mulero, E.J. Noga, Detection of antimicrobial peptides related to piscidin 4 in important aquacultured fish, Dev. Comp. Immunol. 34 (3) (2010) 331–343.
[27] C.M. Press, O. Evensen, The morphology of the immune system in teleost fishes, Fish Shellfish Immunol. 9 (4) (1999) 309–318.
[28] G.A. Davidson, S.H. Lin, C.J. Secombes, A.E. Ellis, Detection of specific and' constitutive' antibody secreting cells in the gills, head kidney and peripheral blood leucocytes of dab(Limanda limanda), Vet. Immunol. Immunopathol. 58 (3–4) (1997) 363–374.
[29] H.K. Strand, R.A. Dalmo, Absorption of immunomodulatingβ(1,3)-glucan in yolk sac larvae of Atlantic halibut, Hippoglossus hippoglossus(L.), J. Fish. Dis. 20 (1) (1997) 41–49.
[30] C. Hellio, A.M. Pons, C. Beaupoil, N. Bourgougnon, Y.L. Gal, Antibacterial, antifungal and cytotoxic activities of extracts from fish epidermis and epidermal mucus, Int. J. Antimicrob. Agents. 20 (3) (2002) 214–219.